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ATTORNEY DOCKET: 41577-296806

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
BROWN ET AL.	
	Art Unit: **1633**
Serial No. **10/780,809**	
	Examiner: **Scott D. Priebe, Ph.D.**
Filed: **February 17, 2004**	
For: **HUMAN HOOKWORM MODEL AND METHOD FOR MAINTAINING HUMAN HOOKWORM IN A NON-HUMAN PRIMATE**	

TRANSMITTAL OF CERTIFIED PRIORITY DOCUMENT

Commissioner for Patents
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Sir:

Pursuant to the Notice of Allowability mailed September 19, 2006, transmitted herewith is the certified copy of Great Britain Patent Application No. GB 0303691.0 filed in the United Kingdom on February 15, 2003. It is believed that the transmittal of the enclosed priority document fulfills the requirements of 35 U.S.C. § 119(b).

If the Examiner believes any informalities remain in the application that may be corrected by Examiner's Amendment, or there are any other issues that can be resolved by telephone interview, a telephone call to the undersigned agent at (404) 815-6500 is respectfully solicited.

Respectfully submitted,

Jamie L. Greene

By: Jamie L. Greene
Reg. No. 32,467

KILPATRICK STOCKTON LLP
1100 Peachtree Street, Suite 2800
Atlanta, Georgia 30309-4530
(404) 815-6500
Our Docket: 41577-296806

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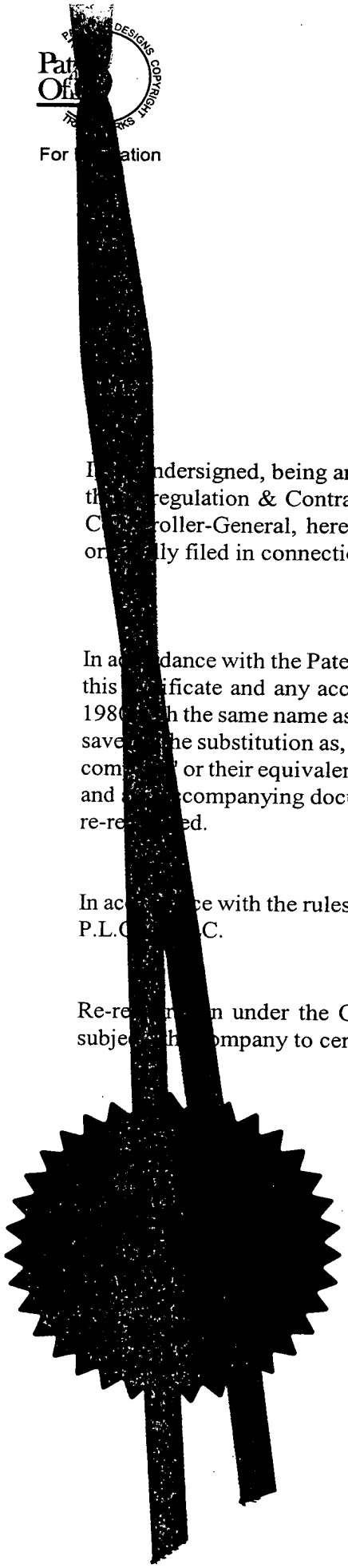

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2. Patent application number *(The Patent Office will fill in this part)* — 0303691.0

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

THE SECRETARY OF STATE FOR DEFENCE
DSTL
Porton Down
Salisbury. Wiltshire. SP4 0JQ

Patents ADP number *(if you know it)* — 6997670005.

If the applicant is a corporate body, give the country/state of its incorporation — GB

4. Title of the invention — Model for Development of Vaccines

9/L
24/2/3
Pm

5. Name of your agent *(if you have one)* — Beckham Robert William

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Country	Piority application number *(if you know it)*	Date of filing *(day / month / year)*

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Claim(s)	3 DML
Abstract	1
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11. I / We request the grant of a patent on the basis of this application.

Signature SSkelton Mr Stephen Skelton Date 13 February 2003

12. Name and daytime telephone number of person to contact in the United Kingdom — Miss Laura Morrison 0117 91 30228

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1. Your reference: P1347

2. Patent application number *(if you know it)*:

3. Full name of the or of each applicant: The Secretary of State for Defence

4. Title of the invention: Model for the Development of Vaccines

5. State how the applicant*(s)* derived the right from the inventor*(s)* to be granted a patent: By virtue of the DSTL named Inventors terms & conditions of employment and Section 39 of the Patents Act 1977 and by virtue of the University of Nottingham Inventors agreement to assign their rights to the Applicant*

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7. I/We believe that the person*(s)* named over the page *(and on any extra copies of this form)* is/are the inventor*(s)* of the invention which the above patent application relates to.

Signature: J C Robbie Date: 15 June 2004

Dr J C Robbie

8. Name and daytime telephone number of person to contact in the United Kingdom: Miss Laura Morrison
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BROWN Alan
University of Nottingham
Nottingham
NG7 2RD
UNITED KINGDOM

Patents ADP number *(if you know it):*

HOOI Doreen S W
University of Nottingham
Nottingham
NG7 2RD
UNITED KINGDOM

Patents ADP number *(if you know it):*

PRITCHARD David Idris
University of Nottingham
Nottingham
NG7 2RD
UNITED KINGDOM

Patents ADP number *(if you know it):*

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Have you signed the form?

Enter the full names, addresses and postcodes of the inventors in the boxes and underline the surnames

GRIFFITHS Gareth David
DSTL
Porton Down
Salisbury
Wiltshire
SP4 0JQ
UNITED KINGDOM

Patents ADP number *(if you know it)*:

PEARCE Peter Colin
DSTL
Porton Down
Salisbury
Wiltshire
SP4 0JQ
UNITED KINGDOM

Patents ADP number *(if you know it)*:

~~**RICE** Paul~~
~~DSTL~~
~~Porton Down~~
~~Salisbury~~
~~Wiltshire~~
~~SP4 0JQ~~
~~UNITED KINGDOM~~

Patents ADP number *(if you know it)*:

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Have you signed the form?

SCOTT Elizabeth Ann Marie
DSTL
Porton Down
Salisbury
Wiltshire
SP4 0JQ
UNITED KINGDOM

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Model for the Development of Vaccines

This invention relates to a method of maintaining a live strain of human hookworm, and a related model for the development of vaccine candidates to protect against hook worm infection and other conditions.

The development of vaccines for use as a treatment or prophylaxis against any one of a wide variety of different conditions is an extremely challenging scientific problem. In humans this is particularly challenging. However, because vaccines have been shown to be very successful at treating or preventing many types of conditions there is an on-going need to identify new vaccine candidates both to improve those vaccines currently on the market but also to treat and protect against conditions for which no vaccine is currently available. One of the key tools required in the development of vaccines is a method of maintaining an on-going and authentic supply of the appropriate vaccine antigen formulation and also a model that can be used to understand immune responses, monitor disease progression or to test potential vaccine candidates. Preferably, such a method and model should exhibit both the complete patency and pathology of the condition following infection, and should also mimic the condition as closely as possible to that seen in a human host.

One particular disease for which there is a need to identify a vaccine candidate is the human hookworm infection *Necator americanus*. It is currently estimated that a billion

people worldwide harbour hookworm infections, making them a leading cause of anaemia and malnutrition, particularly in children and women of child bearing age in developing countries [Chan, M. et al. Parasitology 109: 373-387 (1994); Hotez, P. J. and Pritchard, D. I. Scientific American 272(6) 42-48 (1995); Stephenson, L. Pathophysiology of Intestinal Nematodes. The Geohelminths: Ascaris, Trichuris and Hookworm. C. V. Holland and M. W. Kennedy. Boston, Kluwer Academic Press. 2: 39-61 (2001)]. The infection is considered by many to represent a significant threat to the health and well being of afflicted communities and consequently efforts are being concentrated on developing a full understanding of the molecular biology of the host-pathogen interface, with a view to developing efficacious vaccines to protect against hookworm disease. To this end two major initiatives were recently announced, with the intention of increasing our knowledge of the molecular genetics of hookworms (The Wellcome Trust Beowulf Initiative) and to develop rationally designed vaccines for hookworm infection (The Hookworm Vaccine Initiative, Sabin Vaccine Institute and George Washington University). However one of the major problems with these programmes is that the hookworm lifecycle is difficult to maintain in animal models because of the subtle adaptation of the human hookworm to live in its definitive host. This also has the result that it is difficult to maintain an on-going supply of the hookworm larvae with the same molecular integrity as that which infects humans. As such, to support this work it will be necessary to develop a method of maintaining a supply of human hookworm and also a vaccine model in animals that exhibits the full patency and pathology of human hookworm infection.

Several hookworm models are currently in existence and these were developed to investigate the immunobiology of the human hookworm infection. These include the murine model of human hookworm infection where the adult mouse is infected with the parasite. This model has several problems that include that the adult mouse is unable to retain adequate numbers of larvae in the gut. As a result the model is unable to exhibit either the parasitology or the immunology of the human disease sufficiently accurately to be used as a mimic for the disease as exhibited in humans. It can not therefore be used as a model for the development of either a vaccine or other medicaments.

In addition to the murine hookworm model, a canine model is also in use. Although this model is able to exhibit a form of the disease, the canine host is unfortunately unable to support human hookworm and so the model utilises *Ancylostoma caninum*, the dog hookworm. While such a model is valuable for proof of principle studies, for example with trial vaccines, it clearly has several limitations with respect to use as a model for studying human hookworm for the development of a human hookworm vaccine because it utilises a different hookworm species.

Finally the hamster has been studied for use in work of this type. It has been shown that it is not possible to infect an adult hamster with the disease and therefore this species can not be used as a model as such whereby for example the animal could be pre-vaccinated prior to challenge etc. A further problem also exists in that the model does not respond to L3 larvae, again because of the inability to use adult animals. However, the hamster has proved to be a valuable tool for the maintenance of a hookworm strain in the laboratory. The neonate is infected with the hookworm larvae, the hookworm is

passaged through the animal, collected and then either used for experiementals purposes or re-passaged to maintain further supplies. Hookworm obtained in this manner has proved useful for further understanding the protective inflammatory responses to hookworm challenge following vaccination Ghosh, K. and Hotez, P. The Journal of Infectious Dieases 180: 1674-1681 (1999); Hotez, P. J., et al. Immunological Reviews 171: 163-172 (1999); Liu, S., et al. Vaccine 18: 1096-1102 (2000); Culley, F. J., et al. European Journal of Immunology 32(5): 1376-1385 (2002). However, recently it has been shown that hookworm passaged in this manner does not remain true to the authentic strain of human hookworm. Hence there are several limitations when trying to utilise such material as part of a model for human hookworm for example for development of a vaccine, or other medicaments.

As such to date, there are several problems associated with the known animal models for development of human hookworm vaccines and it would therefore be desirable to develop a well characterised primate model to enable the future vaccine development in a species closely allied to man. Such a model would have several advantages including that it would be able to support the human hookworm infection throughout the whole life cycle, it would provide an on-going source of authentic hookworm causative agent, it would provide a more effective mimic for the immunological response of a human to the hookworm infection and could therefore provide a greater understanding of its pathology, and would also provide a better vehicle for monitoring the efficacy of any vaccine candidates. The model and associated methods could be used to provide materials required during the development programme, test the efficacy and toxicity of desirable vaccine candidates, assess adjuvants, delivery routes and systems, frequency

of inoculation, and to ascertain the immunological phenotype associated with protection with a higher degree of experimental validity than is available to date with the known models.

A method of maintaining human hook worm has now been developed which utilises a primate host. This method has been developed to provide a hookworm model that overcomes the above problems. The method comprises infecting a primate, preferably a marmoset and more preferably *Callithrix jacchus* with non-passaged human hookworm, in this instance a field isolate collected in October 2001 from Papua New Guinea (Haven, Madang Province). The method has demonstrated for the first time that immunogenic patent and pathological infections can be established in a primate. By use of this method a model has been established which demonstrates the patency of the human hookworm infection when infected with fresh field isolate of *Necator americanus* obtained from infected human. This is the first time that a model has been reported which is authentically able to mimic human hookworm infection.

Significantly data obtained from these models indicated that the pathology of the disease was much more severe in those primates that were infected with fresh field isolate when compared to animals infected with hamster adapted hookworm strain. In animals infected with fresh field isolate the haemoglobin levels, packed cell volume and erythrocyte counts were significantly reduced, while those infected with the laboratory strain showed no evidence of this pathology. This indicates that attenuation had likely occurred in the hamster adapted laboratory strain, presumably as a result of repeated passage through hamsters as this strain has been maintained since 1983. These results

further demonstrate that the hamster adapted laboratory strain of human hookworm has several restrictions when used as a mimic for human hookworm infection and is unlikely to be useful as an effective source of hook worm infection for use in a model to assess likely vaccine candidates. As such, development work conducted using hamster source of hookworm infection is likely to be flawed. Interestingly though, one animal infected previously with the laboratory strain and subsequently infected with the field isolate may have been protected from pathology by the attenuated laboratory strain. Further assessment of the infection showed it to be associated with increased total plasma IgE levels and the appearance of specific IgG antibodies to adult worm excretory/secretory (ES) products. This was demonstrated by using ELISA and Western blotting techniques in conjunction with a panel of well characterised anti-human reagents. The appearance of antibodies seemed not be affected by the type of hookworm infection used and in both cases the immune response was reminiscent of that seen in infected patients in endemic areas. Finally, whole blood basophil histamine release was recorded to anti IgE, ES products, and a recombinant hookworm allergen, calreticulin, again in parallel with observations made in human populations. The basophil histamine release to multiple agonists was found to be most consistent in animals exposed to the field isolate, likely reflecting more efficient loading of $Fc_{\varepsilon}RI$ on basophils with parasite-specific IgE, which was not detectable serologically due to the low sensitivity of anti-human IgE reagents in ELISA. Again these results demonstrate the limitations of the currently used hamster adapted laboratory strain of the hookworm infection and the advantages of the present invention.

It is believed that this method provides for the first time a mechanism for maintaining human hookworm infection with the integrity of fresh field isolate. It is believed that

the associated primate model provides, for the first time, a unique opportunity to accelerate the development of a hookworm vaccine in a system where vaccine safety, delivery and efficacy can be assessed against worm establishment, parasite patency, infection associated pathology and immunological characteristics associated with the human hookworm infection. The infection of primates with fresh field isolates of *Necator americanus* will provide an adequate and informative tertiary model for the assessment of adjuvant safety in that the model offers many of the pathological and immunological features associated with hookworm infection of the definitive host. It is also believed that this model will provide for the first time an opportunity to dissect imunologically and physiologically the relationship between parasitic infection and the development of allergenic sensitivity to environmental allergens thus accelerating work to establish any relationship between the apparent protective effects of hookworm infection against the development of respiratory wheeze to dust mites.

It is an object of the present invention to develop a model for the use in the development of vaccines. It is a further object of this invention to develop a primate model that is able to exhibit the patency and pathology of human hookworm. It is another object of this invention to develop a model that can accelerate the development of a human hookworm vaccine. These and other objects of this invention, will become apparent in light of the following disclosure.

Summary of the Invention

According to a first aspect this invention relates to a method of maintaining human hookworm comprising infecting a primate with human hookworm and maintaining the primate.

According to a second aspect this invention relates to a method of obtaining human hookworm larvae comprising maintaining the hookworm according to the present invention and retrieving the hookworm larvae from the faecal material of the primate.

According to a third aspect this invention relates to a method of developing a therapeutic agent comprising utilising human hookworm obtained according to the present invention.

According to a fourth aspect this invention relates to a model for investigating human hookworm comprising a primate infected with human hookworm isolate. This invention also relates to the use of such a model.

Detailed Description of the Invention

All publications cited herein are hereby incorporated by reference in their entirety, unless otherwise indicated.

The elements of the model are described in more detail below.

This invention relates to a method of maintaining human hookworm comprising infecting a primate with human hookworm and maintaining the primate.

The human hookworm is *Necator americanus*. The human hookworm used in this method can be initially obtained from a wide variety of different sources. Preferably the hookworm is a non-adapted human hookworm, either obtained from a reliable source of human hookworm or more preferably the hookworm is fresh human hookworm isolate. The human hookworm isolate used herein was obtained from a fresh *N. americanus* isolate from an infected human. In this instance faecal material was obtained from a hookworm infected individual living in Haven village on the Bogia Coast Road, Madang Province in October 2001, and was cultured as previously described Harada, Y. and Mori, O. Yonago Acta Medica 1: 177-179 (1955). Freshly cultured larvae were used to infect marmosets (Dstl, Proton Down, UK). As a control this larvae was also used to infect neonate hamsters (University of Nottingham, UK).

This method comprises infecting a primate with the human hookworm infection. Preferably the primate used is a marmoset, more preferably *Callithrix jacchus*. Common marmosets (3 male and 3 female, bred at CBD Porton Down) weighing 319-516g were used. These were maintained as mixed sex pairs where the males had been vasectomised. Each pair of primates were housed in 4 stainless steel cage units measuring H72 x W47 x D60 cm connected together by 2 horizontal external extensions and 1 vertical extension (H18 x W71 x D23 cm and H105 x W 17 x D 23 cm respectively) in order to allow full use of all 4 units. The cages comprised various items

of cage furniture, including hanging wooden dowels, buckets and other playthings, were also placed in the cages. Illumination was provided by sodium lighting, at a level of 350-400 lux 1 m from the ground, using a 12 hour light/dark cycle with dusk and dawn effects over 1 hour periods. The primates were maintained on a daily diet of 20g pellets (SDS primate diet CPDE) with supplements of orange segments. Additional supplements included banana, apple and egg. The primates were additionally fed from a tray hanging below a horizontal extension on their cage which was filled with sawdust in which a small amount of preferred foods, such as raisins, was dispersed such that the primates could engage in foraging behaviour. Water was available ad-libitum. During behavioural training and testing the pairs were separated so that each had use of a single upper unit of its home cage with a rigid extension unit (H18 x W17 x D30 cm) attached to the front.

The primates were infected with the hookworm isolate, preferably using transcutaneous infection. In one method, marmosets were anaesthetised with ketamine (15 mg per animal) and an area of skin approximately 2 cm^2 just below the scapula shaved. Infective larvae were placed on a gauze fixed to a self adhesive horse bandage (International Market Supply, Dane Mill, Broadhurst Lane, Congleton, Cheshire, CW12 1LA). The bandage was subsequently wrapped around the thorax and held in place with a tubigrip jacket. The jacket and bandage were removed after 24 h. The primates were infected with from about 200 to about 1000 larvae respectively.

The infected primates were maintained under study for a period of 3 to 12 months following infection. During this time primates were routinely observed to ensure that

they were taking food and water. They were also observed in case of the occurrence of any specific adverse signs, for example lethargy or problems of a respiratory nature following infection, and the like. Blood samples were taken twice monthly in order to monitor specific antibody formation, red blood cell count, haemoglobin level and packed cell volume. These were used both as a source of information regarding the progress of the infection and also, as the infection progressed, to ensure that the animals were not in danger of adverse side effects due to blood loss. All blood samples were taken in accordance with recommended animal welfare guidelines ("Removal of blood from laboratory mammals and birds". Laboratory Animals (1993) 27, 1-22).

This invention also relates to a method of obtaining human hookworm larvae comprising maintaining the hookworm according to the present invention and retrieving the hookworm larvae from the faecal material of the primate using standard isolation techniques for example as described in Carr A. and Pritchard D. I., Parasite Immunology 9 219-234 (1987). Human hookworm obtained in this manner can have many uses. For example this method could be used to provide an on-going sustainable source of human hookworm which maintains the patency, pathology and immunological characteristics of human hookworm in a human host. In addition human hookworm obtained in this manner could be used in a method to develop a therapeutic agent by any one of standard developmental techniques known to one skilled in the art. A specific example of a therapeutic agent that such hookworm could be used to develop is a vaccine, more preferably a human hookworm vaccine. Such a development method would provide several advantages over known development methods utilising known

neonate adapted hookworm since the hookworm would exhibit the patency, pathology and immunology of human hookworm.

This invention also relates to a model for investigating human hookworm comprising a primate infected with human hookworm isolate. In particular this model is one wherein the model itself exhibits the patency, pathology and immunology of the hookworm infection essentially as that exhibited in human host. This model provides several advantages over the hookworm models already known. These include that the hookworm is a wholly representative model of human hookworm in a human host since it comprises a host which is known to be very similar to a human which has been infected with the human hookworm itself. In addition, due to the similarities between the primate and the human, the rate of the adaptation of the human hookworm, if at all, will be much slower in a primate host than in a murine host. This has the effect that the human hookworm retains the same integrity as displayed in a human host for a much longer period of time. Such a model has many uses in the scientific areas of developing an understanding of the hookworm infection but also in developing new methods of treatment and prevention of the hookworm infection and related conditions. Examples of specific and important uses of such a model include, but are not limited to, the development of a hookworm vaccine, for the investigation into the immunological response to human hookworm, for the development of a greater understanding of the relationship between the hookworm and allergenic sensitivity to environmental allergens, and for the development of a vaccine for protect against the development of respiratory conditions.

Experimental Results

In order to ascertain the success of the methods and models disclosed herein several experiments were conducted to compare the hookworm obtained by the method of this invention, ie marmosets infected with human hookworm isolate, with a hookworm obtained from a control model, marmosets infected with hamster adapted strain of human hookworm, ie the hookworm strain typically used in laboratories today.

The control primates were infected with hamster adapted laboratory strain of *Necator americanus*. The strain was originally obtained in 1983 from Dr G. Rajasekariah of Hindustan, CIBA-GEIGY Ltd., Bombay, India and has been maintained in syngeneic DSN hamsters (*Mesocricetus aureus*) at the University of Nottingham since that date (Pritchard D. I. et al Parasite Immunology 8 359-367 (1986)). The laboratory strain has been maintained as follows. 2-4 day old neonate hamsters are infected percutaneously with 100 infective third stage larvae and the infection allowed to proceed until adult worms in the small intestine became fecund approximately 42 days post-infection. To obtain fresh infective larvae, faecal material containing *N. americanus* eggs are cultured by a method described by Kumar, S. and Pritchard, D. I. International Journal For Parasitology **22**(5): 563-572 (1992). Faecal material is mixed with activated charcoal, 1 % (w/v) amphotericin B (final concentration) and water to form a smooth paste, which is applied to the upper half of a 5 x 30 cm strip of filter paper. The strips are then suspended in a large glass chromatography tank containing approximately 750 ml of distilled water. The tanks are sealed and incubated at 28 °C for 7-10 days, after which the filter paper strips carefully removed and discarded. The water containing the larvae is transferred to a measuring cylinder and the larvae allowed to settle for 2 h. After this

period the water is aspirated off and the larvae washed twice to remove any faecal contamination. Finally, washed larvae are re-suspended and stored in distilled water until required. The strain used in these experiments has been passaged approximately 460 times through DSN hamsters since the time that it was initially obtained from Ciba Geigy in 1983. The control primates were infected with the hamster adapted laboratory strain of *Necator americanus* as described above.

The primates were infected as follows:

Primates 1 and 2 were infected with 300 laboratory strain larvae and re-infected with 300 laboratory strain larvae on day 103.

Primate 3 was infected with 300 laboratory strain larvae and re-infected with 300 laboratory strain larvae on day 98.

Primate 4 was infected with 300 laboratory strain larvae and re-infected with 300 human strain larvae on day 98.

Animals 5 & 6 were infected with 300 and 600 human strain larvae respectively.

The following were monitored in order to assess the primate model:

- Egg production which was assessed using salt floatation Keymer A. E. et al Parasitology **101**: 69-73 (1990);
- Haemoglobin levels (normal range 14.9-17.9g/dl), erythrocytes (normal range 5.7-6.95 x 10^{12}/l), and mean erythrocyte cell volume (MCV, normal range 48-87 fl) were measured using a Baker 9000 haematology analyser;

- Packed cell volume was measured using a Hawksley haematocrit centrifuge and reader (normal range 0.42-0.52)
- Peripheral blood leucocytes were measured using a Baker 9000 haematology analyser (normal range 7.3 x 10^9/l).

Further studies were also conducted to assess the human hookworm infection compared to that of the control hamster adapted laboratory strain.

Adult *N. americanus* excretory/secretory (ES) products were collected as described by Brown, A. and Pritchard, D. I. Parasite Immunolgy **15**: 195-203 (1993). *N. americanus* infected hamsters were killed 35 days post-infection and the small intestine removed, cut along its length and placed in a petri dish containing Hanks buffered saline solution (HBSS). The petri dishes were incubated at 37 °C to allow the adult worms to detach voluntarily from the intestine thus minimising the possibility of host tissue contaminating subsequent ES cultures. Detached adult worms were washed extensively in RPMI 1640 containing 100 i.u./ml penicillin and 100 µg/ml streptomycin over a period of 2 h followed by further culture in RPMI 1640 for 24 h. ES products obtained after 24 hours were stored at –20 °C until required.

The proteolytic activity of normal and heat inactivated ES products was determined using fluorescein isothiocyanate labelled casein (FITC-casein) as described by Beynon R. J. and Bond, J. S. Proteolytic enzymes-A practical approach. Oxford, IRL Press 1989. 12 µg of ES (20 µl) products were mixed with 10 µl of FITC-casein (stock 0.5

mg/ml) and 170 µl of 50 mM phosphate buffer, pH 6.5 containing 5 mM cysteine and incubated at 37 °C for 2 h. To stop the reaction and precipitate any undigested protein 120 µl of 5 % w/v trichloroacetic acid was added and the tubes allowed to stand at room temperature for 1 h. Precipitated was protein removed by centrifugation at 13 000 g for 10 minutes. Triplicate, 20 µl aliquots of the supernatant were added to 80 µl of 0.5 M Tris pH 8.5 and the fluorescence measured (excitation 490 nm, emission detection 525 nm) using a Dynex MFX microplate fluorimeter.

The expression and purification of recombinant calretiulin was conducted as follows. *E. coli* M15 (pREP4) transformed with the plasmid pQE-CalΔSig which encodes *N. americanus* calreticulin deleted by PCR of the N-terminal signal sequence and fused to 6x histidine tag to facilitate affinity purification Pritchard, D. I., et al. Parasite Immunology **21**: 439-450 (1999) were grown overnight at 37 °C in LB broth containing kanamycin (30 µg/ml) and ampicillin (200 µg/ml). The culture was diluted 1:7 in fresh medium and, after 30 min, IPTG was added to a final concentration of 2.8 mM. After vigorous shaking for 3 hours at 37 °C, the culture was harvested by centrifugation.

Recombinant calreticulin was purified using a a combination of 'Bugbuster' protein extraction reagent (Novagen) and a His Bind Purification kit (Novagen). Harvested cells were resuspended in Bugbuster reagent (5 ml per gram of cell pellet) containing 25 units benzonase per ml of Bugbuster reagent and incubated at room temperature for 20 min. Insoluble cell debris was removed by centrifugation at 16000 g for 20 min at 4 °C and the supernatant loaded directly onto a 5 ml His bind resin column previously equilibrated with 5 column volumes of 50 mM $NiSO_4$ followed by 3 column volumes of

binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9). Following application the column was washed with 10 volumes of binding buffer and 6 volumes of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH7.9 prior to elution. Bound calreticulin was eluted with 6 column volumes of elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-Cl, pH 7.9). Fractions containing calreticulin as determined by protein estimation (BioRad) were pooled, dialysed against PBS and stored at –20 °C until required. The purified recombinant protein was sequenced by MALDI-TOF mass spectrometry to confirm its identity.

The immunology was investigated by determining both the total IgE response and the specific IgG response in each of the primates. The total IgE response was determined by coating a 96 well polystyrene plate with 50 µl of a mouse anti-human IgE (BD Pharmingen, 5 µg/ml diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6) overnight at 4 °C. The plates were washed with phosphate buffered saline/0.05 % Tween 20, pH 7.2 (PBS/Tween) and blocked with 200 µl of 1 % bovine serum albumin in PBS (BSA/PBS) for 1 h at room temperature. After blocking, the plates were washed again and 50 µl of marmoset serum (diluted 1:5 in 1 % BSA/PBS) added to each well and incubated overnight at 4 °C. In addition 50 µl of human IgE standards (doubling dilutions from 100 ng/ml to 1.56 ng/ml) were included on each plate. All assays were carried out in duplicate. Following overnight incubation the plates were washed again and 50 µl of biotinylated mouse anti human IgE (2 µg/ml diluted in 1 % BSA/PBS) added to each well and incubated at room temperature for 2 h. After 2 h the plates were washed again and 50 µl of streptavidin conjugated to horseradish peroxidase (diluted 1:1000 in 1 % BSA/PBS) added to each well and incubated for 1 h at room temperature.

The plates were washed one final time and developed with 100 µl TMB (0.1 mg/ml) containing 6 µl of hydrogen peroxide per 10 ml of TMB. The reaction was stopped by adding 20 µl of 2.5 M sulphuric acid and the absorbance of each well measured at 450 nm.

The specific IgG response was determined by coating a 96 well polystyrene plate with 50 µl of *N. americanus* ES products (5 µg/ml in 0.05 M sodium carbonate/bicarbonate buffer pH 9.6) and incubating at 4 °C overnight. The plate was washed with PBS/Tween and the wells blocked with 200 µl of 5 % skimmed milk powder/PBS for 1 hr at 37 °C. The plate was washed as before and 50 µl of marmoset serum, diluted 1:100 in skimmed milk powder/PBS added to individual wells and the plate incubated at 4 °C overnight. The plate was washed again and 50 µl of sheep anti human IgG (Binding Site) diluted 1:1000 in blocking agent was added to individual wells and the plate incubated for 2 h at room temperature. The plates were washed again and antibody binding visualised by the addition of 100 µl TMB prepared as described above. The reaction was stopped by adding 20 µl of 2.5 M sulphuric acid and the absorbance of each well measured at 450 nm. All assays were carried out in duplicate. ELISA values are expressed as the absorbance at 450 nm after the subtraction of a negative control.

Sera from infected marmosets were also analysed by Western blotting. *N. americanus* ES products (10 µg/lane) were separated, under reducing conditions, by 12 % SDS-PAGE Laemmli, U. K. Nature **227**: 680-685 (1970) and transfered onto a nitrocellulose

membrane Towbin, H., Staehelin, T. and Gordon, J. Proceedings of the National Academy of Science **76**(9): 4350-4354 (1979). Western blots were blocked for 1 h in 5 % skimmed milk powder in TBS at room temperature. Marmoset serum (diluted 1:200 in 5 % skimmed milk powder/TBS) was added to the blots and incubated overnight at 4 °C. Blots were washed with TBS/0.05 % Tween 20 and then incubated in sheep anti-human IgG (Binding Site) diluted 1:1000 in 5 % skimmed milk powder/TBS for 2 h at room temperature. Following washing blots were developed in chloronapthol (10 mg/ml) containing 30 µl of hydrogen peroxide.

Furthermore, whole blood basophil collected from the animals was challenged for their ability to release histamine. 100 µl of whole blood was collected from the primates into heparinised tubes and the volume was made up to 500 µl using Pipes buffer (0.01 M Piperazine-N'N-bis[2-ethaneculfonic acid], 0.14 M sodium acetate, 5 mM potassium acetate, 0.1 % glucose, 1 mM $CaCl_2$ and 0.03 % human serum albumin, pH 7.4). Spontaneous histamine release was assessed following incubation for 1 h at 37 °C, while total histamine release was assessed when 50µl of whole blood in 450 µl dH_2O was freeze-thawed three times. Standard histamine calibrators of 0, 10, 25, 50, 100 and 250 ng/ml (Hycor Biomedical Ltd, Penicuik, UK) were included with each set of whole blood challenges, mediated by anti-IgE, ES products or recombinant calreticulin. Histamine released in each whole blood challenge was detected using a Histamine Assay Kit (Hycor Biomedical Ltd.). 50 µl of challenged whole blood was added to histamine-coated 96 wells followed by 50 µl of mouse anti-histamine monoclonal conjugated to alkaline phosphatase. Following an incubation of 1 h at room temperature wells were washed three times with a provided EIA wash solution.

Antibody binding was visualised by the addition of 100 µl of 1 mg/ml of p-nitrophenyl phosphate (pNPP) substrate The plates were developed for 1 h at 37 °C and the absorbance measured at 405 nm using a Dynex MRX absorbance microplate reader plate reader. For all samples and calibrators the percentage binding was determined by the following,

$$\text{Percentage binding} = 100 \times \frac{\text{Absorbance of Sample}}{\text{Average Absorbance of Zero Calibrator}}$$

A standard curve was constructed by plotting the percentage binding against concentration for each histamine calibrator and the levels of histamine in each challenge determined from this curve.

Results

Figure 1 shows the egg production, haemoglobin levels and packed cell volume of each different primate over time.

Figure 2 shows the change in peripheral blood leucocytes and erythrocytes along the time course of infection in Primates 1-4.

Figure 3 shows the change in peripheral blood leucocytes, erythrocytes and mean erythrocyte cell volume along the time course of infection in Animals 5 & 6.

Figure 4 shows the results from ELISA and Western blot analysis of the antigenicity of the ES products of *N. americanus* probed with post-infection marmoset plasma followed by anti-human IgG. For comparison, the peak day of antigenicity as assessed by band intensity on Western blot and peak egg output are indicated below.

Lane 1. Animal 1, Peak antigenicity day 70-84 Peak epg day 70-112.

Lane 2. Animal 2, Peak antigenicity day 70 Peak epg day 84/140.

Lane 3. Animal 3, Peak antigenicity day 75 Peak epg day 47-61.

Lane 4. Animal 4, Peak antigenicity NA Peak epg NA.

Lane 5. Animal 5 Peak antigenicity day 33-123 Peak epg day 48-103.

Lane 6. Animal 6 Peak antigenicity day 33-123 Peak epg day 48-81.

Figure 5 shows Basophil histamine release in infected animals following challenge with anti IgE, anti IgG, *N. americanus* excretory/secretory (ES) products (intact or heat inactivated (HI) to neutralise enzymatic activity) and a recombinant hookworm allergen, calreticulin (recCAL). Histamine release was measured using was detected using a Histamine Assay Kit (Hycor Biomedical Ltd.) as described in the materials and methods.

These results demonstrate that 5 from 6 animals exposed to hookworm larvae demonstrated evidence of infection as indicated by the appearance of eggs in faeces (figure 1). These results also demonstrate that all the animals reacted to infection immunologically, yet only animals exposed to the recently acquired field isolate demonstrated infection-associated pathology, with a dramatic reduction in haemoglobin (Hb) and packed cell volume (PCV) 48-62 days post infection. It is possible that that animal 4 (figure 1, panel B) was protected against infection by the new field isolate by prior infection to what appears to be an attenuated laboratory strain. The dramatic fall

in haemoglobin and PCV levels, accompanied by evidence of a microcytic anaemia, presumably as a direct result of blood loss in the lungs during transit by infective larvae, and feeding by adult worms in the gut (Girod, N., Brown, A. P., Billett, E. E. and Pritchard, D. I. International Journal for Parasitology. In Press (2003)) in animals 5 & 6 when compared to animals 1-4 likely indicates that the hamster adapted laboratory strain had become attenuated and was therefore no longer able to exhibit the full pathology of the infection in the primates. This is reflected by the fact that the Necator adult secretions from the adapted strain have lost the ability to inhibit human platelets platelet aggregations as demonstrated according to Furmidge B. A. et al. Parasitology 112 81-87 (1995).

The results shown in Figures 2 & 3 demonstrate that the erythrocyte numbers in animals 5 and 6 were reduced during infection reaching their lowest levels 40-60 days post-infection, corresponding to peak egg output (figure 3) but no such similar result was seen with animals 1-4 (figure 2). Similarly leucocyte numbers in animals 5 and 6 were seen to increase during infection with the new field isolate but again no such increase was observed in animals 1-4 (figure 2). However, interestingly, animal 4 when re-infected with the new field isolate showed an increase in leucocyte numbers following re-infection (figure 2, panel B). It was also noticeable that animal 5, exposed to the new field isolate alone, in addition to showing evidence of pathology as assessed by PCV and haemoglobin levels also exhibited evidence of microcytic anaemia, in that infection with the new isolate had a significant effect on the mean erythrocyte cell volume (figure 3a).

In all cases the antigenicity of infection was confirmed by the appearance of specific IgG antibodies in ELISA to *N. americanus* ES products (figure 4a-c). Antibodies recognised the classical hookworm 33kDa antigen (as described in Carr A. and Pritchard D. I., Parasite Immunology 9 219-234 (1987)) on Western blots (figure 4d). Although the response was stronger in some individuals than others the peak response on Western blots corresponded in most cases with peak egg output.

As a result of the characteristic elevation of total IgE levels by hookworm infection, the sensitisation of basophils with hookworm specific IgE was also investigated. It can be seen from figure 5a that 5 out of 6 animals demonstrated significant histamine release to ES products and a recombinant hookworm allergen calreticulin. To control for the possible non-specific release of histamine by enzymes in ES products (Phillips C. et al. Journal of Leucocyte Biology (2003) in press) some preparations were heat inactivated by boiling for 30 minutes to neutralise activity (untreated ES released 2439 ± 66.1 fluorescence units over 2 h, no activity was detected in heat inactivated ES products). Significantly, heat inactivated ES products produced a similar level of release, indicating the presence of allergenic material in ES products. To control for the ability of basophils to release histamine, cells were also challenged with anti IgG and anti IgE (figure 5b). 3 out of 6 animals released histamine to anti IgE to a greater extent than that induced by anti IgG. The failure of animals 1, 2 and 4 to release histamine would suggest that the cells were not sufficiently sensitised with hookworm-specific IgE (animals 1, 2 and 4 show the lowest levels of plasma IgE (table 1)), or that a degree of receptor blockade was operating.

In addition the pathology of the infected animals was also investigated. An autopsy was conducted on each of the animals (333 days post-infection, marmosets 1 and 2; 235 days, marmosets 3 and 4; and 137, marmosets 5 and 6). The small intestines were removed from each marmoset, opened along their length and placed in Hanks saline at 37 °C to allow any remaining worms to detach. No residual worms were observed in marmosets 1-4, nine worms were observed in marmoset 5 - these consisted of 4 males, 2 females and 3 worms which were fixed 'in situ' for histology and unable to be accurately sexed. 11 worms were observed in marmoset 6 comprising of 5 males, 2 females and 4 fixed 'in situ' of undetermined sex.

Claims

1. A method of maintaining human hookworm comprising infecting a primate with human hookworm and maintaining the primate.

2. A method according to Claim 1 wherein the human hookworm is *Necator americanus*.

3. A method according to Claim 2 wherein the human hookworm is non-adapted human hookworm, preferably fresh human hookworm isolate.

4. A method according to any of Claims 1 to 3 wherein the primate is a marmoset, preferably *Callithrix jacchus*.

5. A method according to any of Claims 1 to 4 wherein the primate is infected with from about 200 to about 1000 larvae.

6. A method according to any of Claims 1 to 5 wherein the primate is infected with the hookworm transcutaneously.

7. A method of obtaining human hookworm larvae comprising maintaining the hookworm according to Claim 1 and retrieving the hookworm larvae from the faecal material of the primate.

8. A method of developing a therapeutic agent comprising utilising human hookworm according to Claim 1.

9. A method according to Claim 8 wherein the therapeutic agent is a vaccine, preferably a human hookworm vaccine.

10. A model for investigating human hookworm comprising a primate infected with human hookworm isolate.

11. A model according to Claim 10 wherein the model exhibits the patency, pathology and immunology of the hookworm infection in a similar manner to that exhibited in human host.

12. Use of a model according to Claim 10 to develop a therapeutic agent, preferably a hookworm vaccine.

13. Use of a model according to Claim10 to investigate the immunological response to human hookworm.

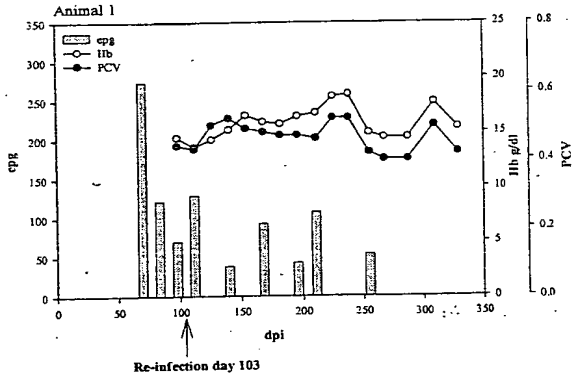
14. Use of a model according to Claim 10 to understand the development of allergenic sensitivity to environmental allergens.

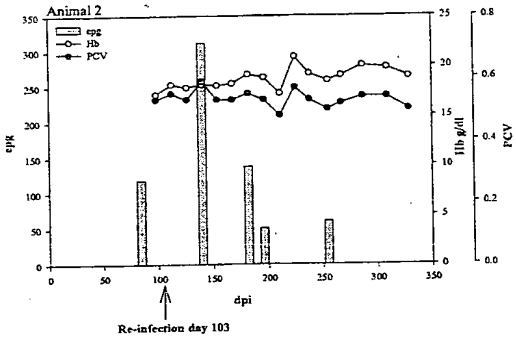
15. Use of a model according to Claim 10 to develop a vaccine for protect against the development of respiratory conditions.

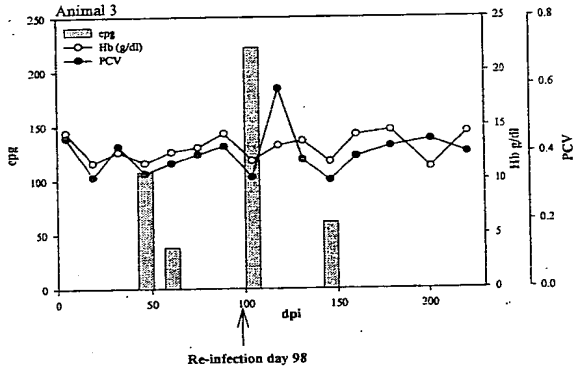
Abstract

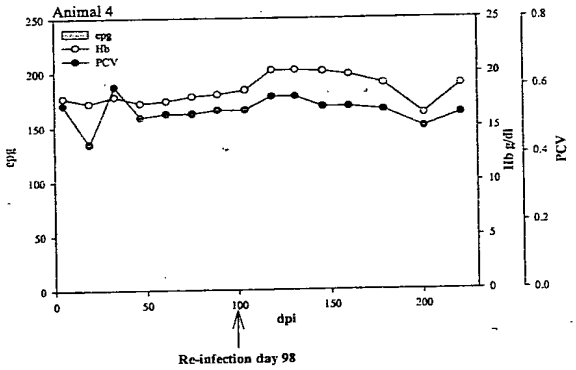
This invention relates to a method of maintaining human hookworm comprising infecting a primate with human hookworm and maintaining the primate and a related method for retrieving human hookworm larvae and use thereof. This invention also relates to a model for investigating human hookworm comprising a primate infected with human hookworm isolate and use thereof.

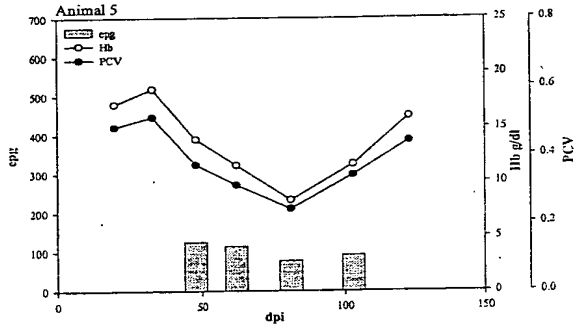
Figure 1


Animal 1
epg
Hb
PCV
dpi
Re-infection day 103


Animal 2
epg
Hb
PCV
dpi
Re-infection day 103


Animal 3
epg
Hb (g/dl)
PCV
dpi
Re-infection day 98


Animal 4
epg
Hb
PCV
dpi
Re-infection day 98


Animal 5
epg
Hb
PCV
dpi

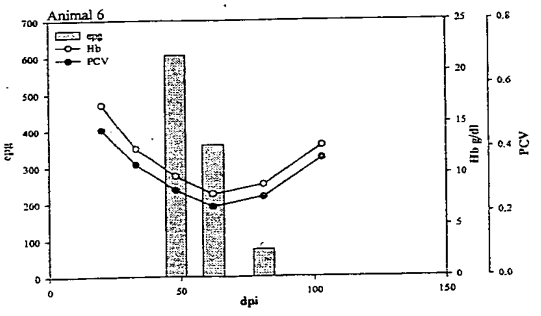
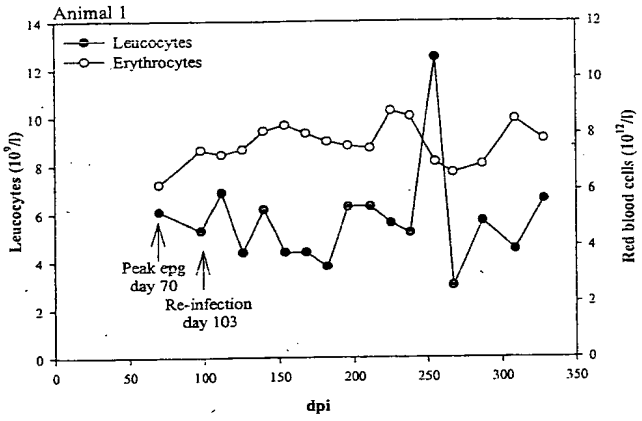
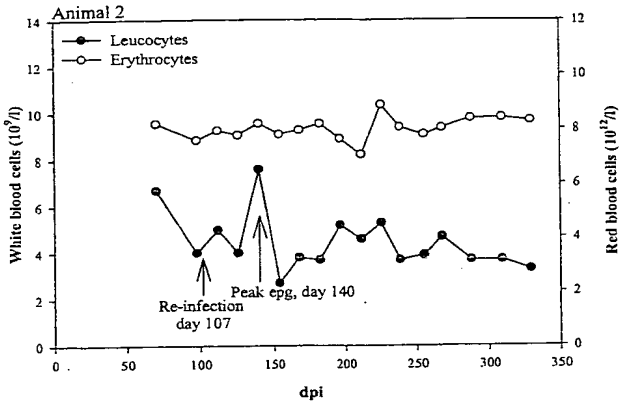
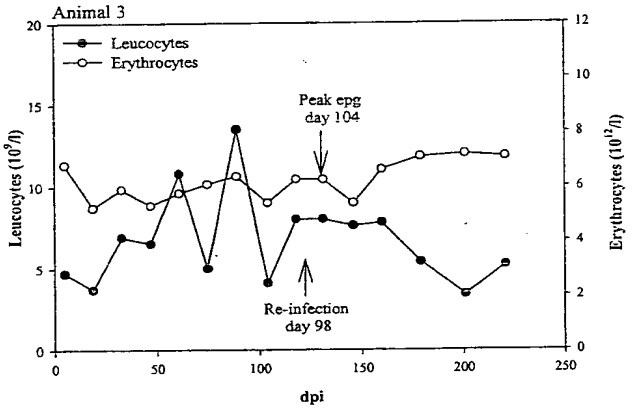

Animal 6
epg
Hb
PCV
dpi

Figure 2


Animal 1
Leucocytes
Erythrocytes
Leucocytes (10^9/l)
Red blood cells (10^{12}/l)
Peak epg
day 70
Re-infection
day 103
dpi


Animal 2
Leucocytes
Erythrocytes
White blood cells (10^9/l)
Red blood cells (10^{12}/l)
Re-infection
day 107
Peak epg, day 140
dpi


Animal 3
Leucocytes
Erythrocytes
Leucocytes (10^9/l)
Erythrocytes (10^{12}/l)
Peak epg
day 104
Re-infection
day 98
dpi

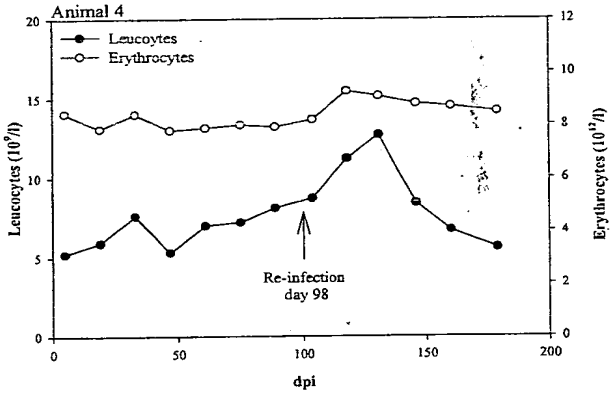
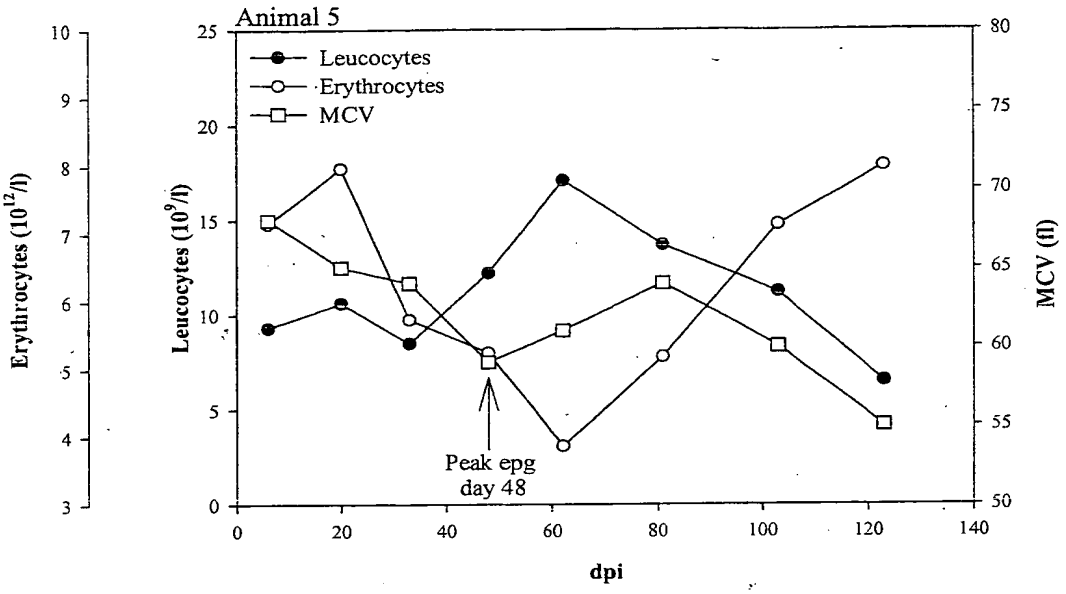

Animal 4
Leucocytes
Erythrocytes
Leucocytes (10^9/l)
Erythrocytes (10^{12}/l)
Re-infection
day 98
dpi

Figure 3


Animal 5
Leucocytes
Erythrocytes
MCV
Peak epg
day 48
Erythrocytes (10^{12}/l)
Leucocytes (10^9/l)
MCV (fl)
dpi

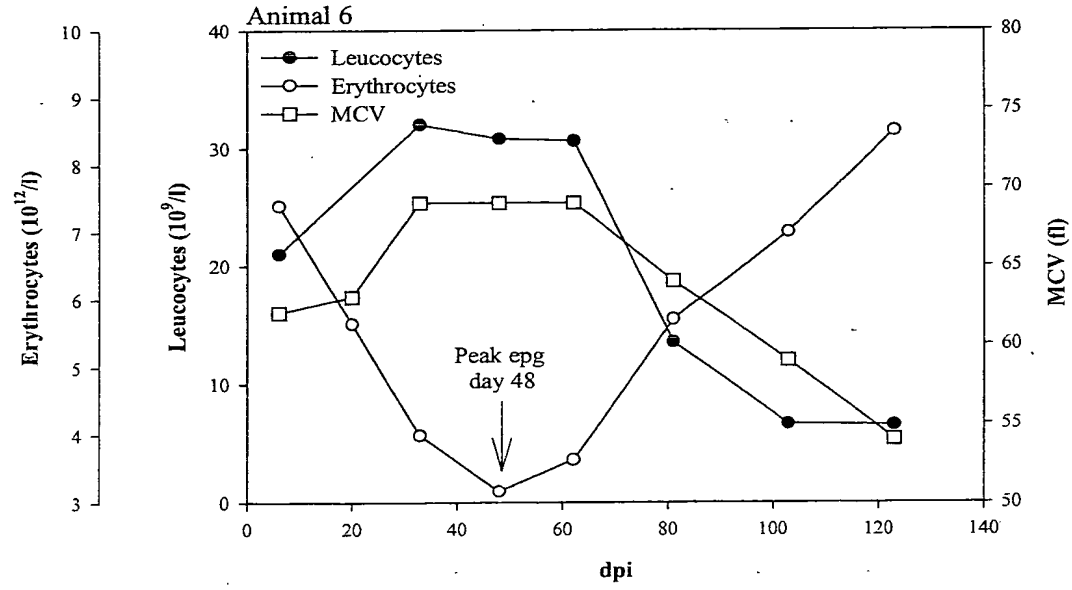

Animal 6
Leucocytes
Erythrocytes
MCV
Peak epg
day 48
Erythrocytes (10^{12}/l)
Leucocytes (10^9/l)
MCV (fl)
dpi

Figure 4

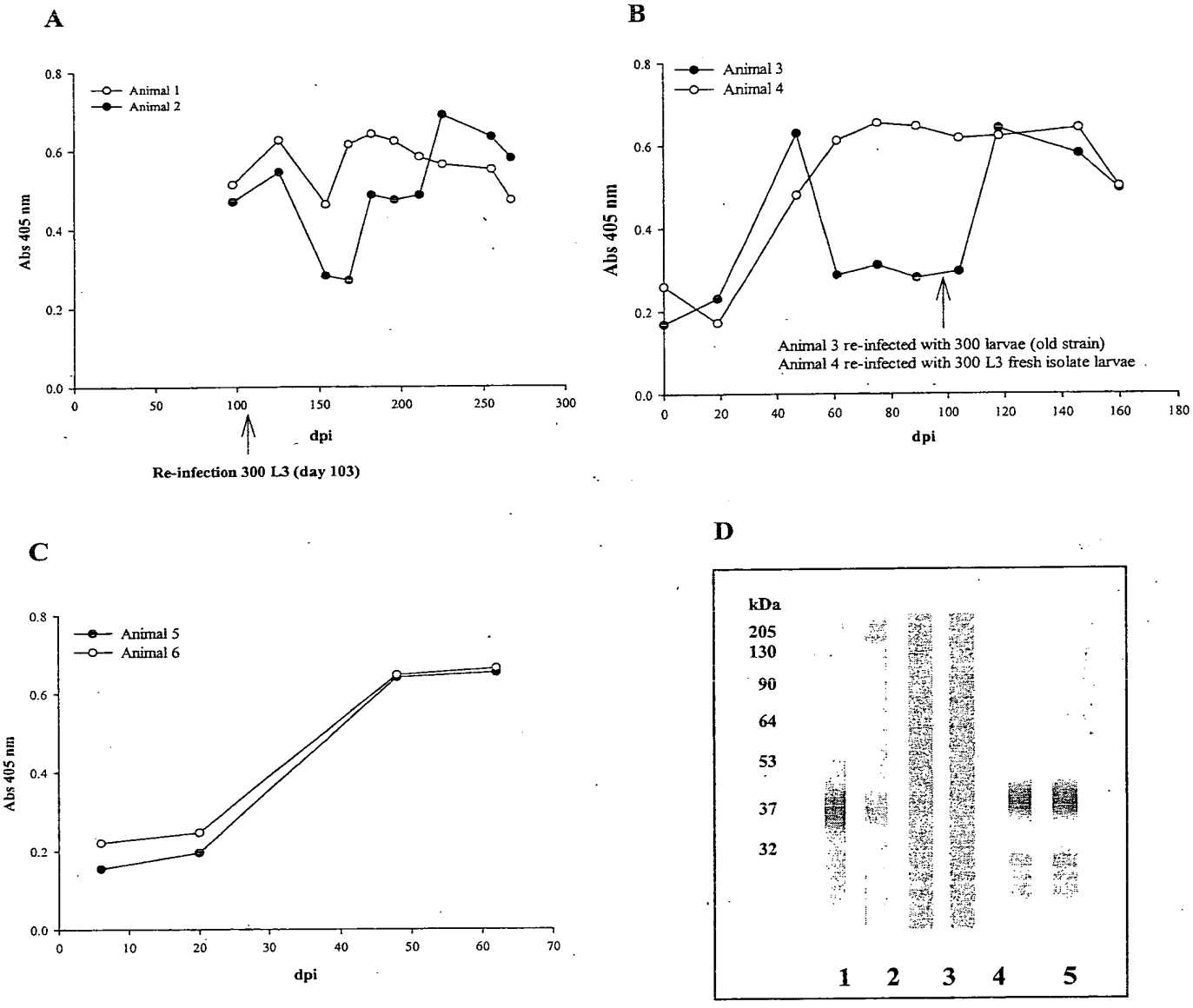

A
Animal 1
Animal 2
Abs 405 nm
dpi
Re-infection 300 L3 (day 103)
B
Animal 3
Animal 4
Abs 405 nm
dpi
Animal 3 re-infected with 300 larvae (old strain)
Animal 4 re-infected with 300 L3 fresh isolate larvae
C
Animal 5
Animal 6
Abs 405 nm
dpi
D
kDa
205
130
90
64
53
37
32
1
2
3
4
5

Figure 5A

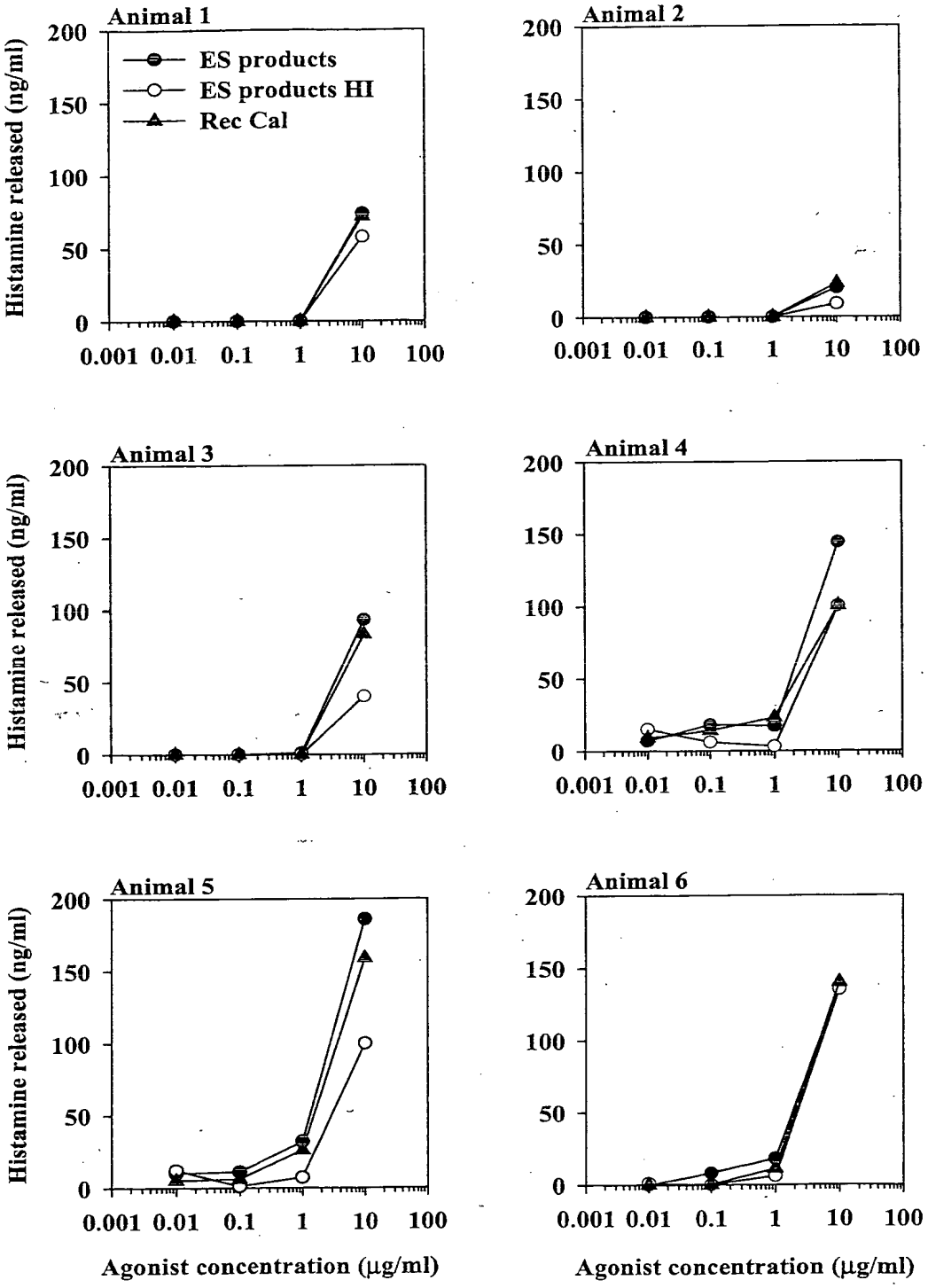
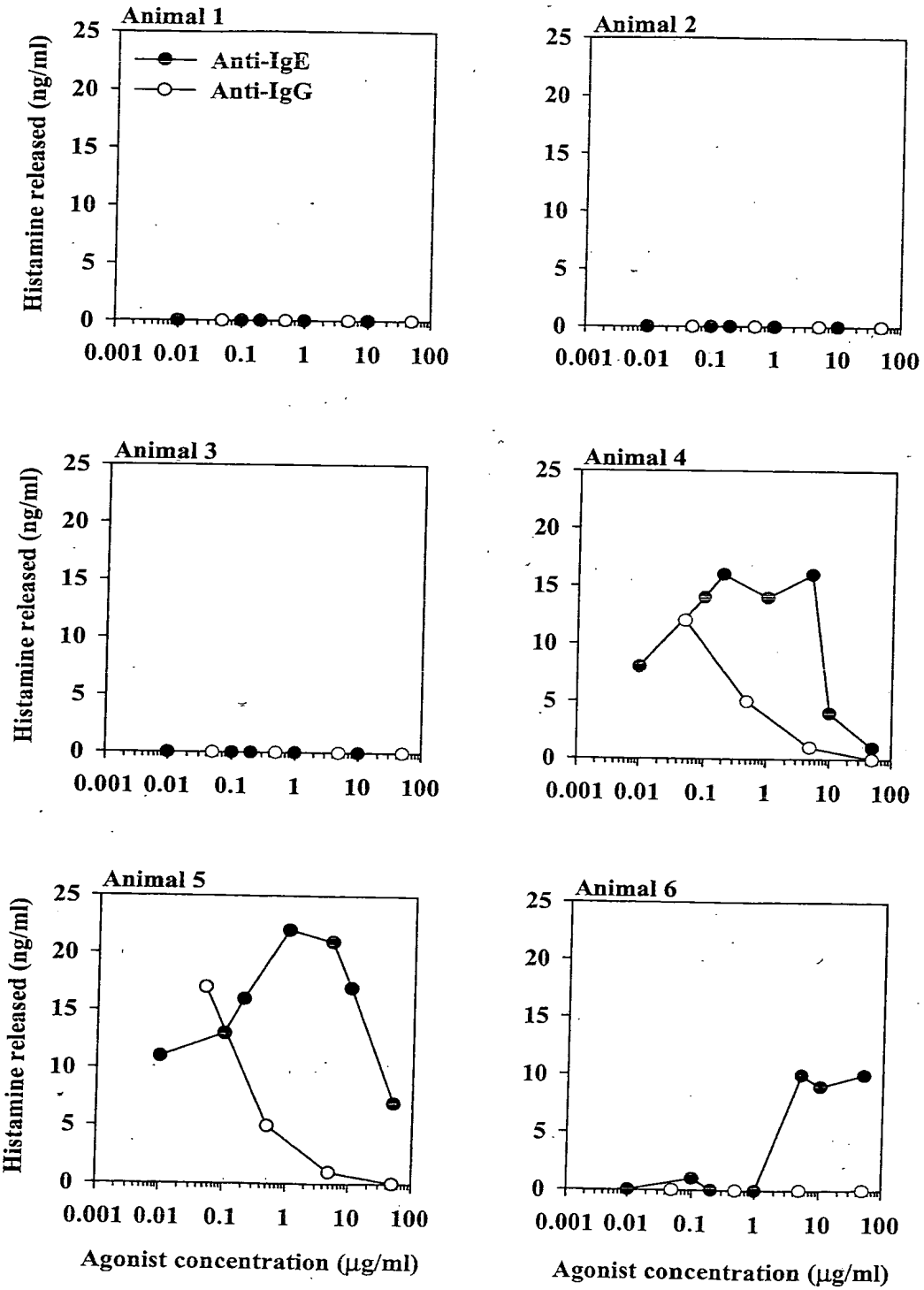

Animal 1
Animal 2
Animal 3
Animal 4
Animal 5
Animal 6
ES products
ES products HI
Rec Cal
Histamine released (ng/ml)
200
150
100
50
0
0.001 0.01 0.1 1 10 100
Agonist concentration (µg/ml)

Figure 5B


Animal 1
Anti-IgE
Anti-IgG
Histamine released (ng/ml)
25
20
15
10
5
0
0.001 0.01 0.1 1 10 100
Animal 2
Animal 3
Animal 4
Animal 5
Animal 6
Agonist concentration (µg/ml)